Transmembrane TNF (pro-TNF) is palmitoylated

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Abstract Human transmembrane tumor necrosis factor (pro-TNF) was examined for protein acylation. The cDNA encoding pro-TNF was expressed in both COS-1 cells and Sf9 cells and metabolic labeling with [^{3}H]myristic or [^{3}H]palmitic acid was attempted. The 17 kDa mature TNF secreted from the transfected cells was not labeled, whereas the 26 kDa pro-TNF was specifically labeled with [^{3}H]palmitic acid. The [^{3}H]palmitic acid labeling of pro-TNF was eliminated by treatment with hydroxylamine, indicating that the labeling was due to palmitoylation of a cysteine residue via a thioester bond. Site-directed mutagenesis of the two cysteine residues residing in the leader sequence of pro-TNF demonstrated that palmitoylation of pro-TNF occurs solely at Cys-47, located at the boundary between the transmembrane and cytoplasmic domains of pro-TNF. Thus, pro-TNF interacts with the plasma membrane via both its proteinaceous transmembrane domain and a lipid anchor.

Key words: Tumor necrosis factor; Transmembrane tumor necrosis factor; Protein palmitoylation; Protein acylation; Post-translational modification; Signal transduction

1. Introduction

Tumor necrosis factor (TNF) is an extremely pleiotropic pro-inflammatory cytokine with a wide range of biological effects [1]. This protein is initially synthesized and expressed as a biologically active transmembrane prohormone (pro-TNF), linked to the membrane via the leader sequence. Synthesis of TNF begins with an unusually long 76 amino acid propeptide sequence. This leader sequence does not serve as a typical N-terminal signal sequence during processing and intracellular targeting [2]. Rather, the leader sequence functions as a signal-anchor sequence, and synthesis and insertion into the endoplasmic reticulum (ER) membrane occur cotranslationally. Then, probably via Golgi-mediated transfer, TNF appears on the plasma membrane as a trimeric, type II transmembrane protein [3]. Finally, pro-TNF cleavage occurs at the outer surface of the plasma membrane, probably by a metallo-proteinase [4,5], and/or other enzyme to release mature, soluble trimeric TNF.

The transmembrane form of TNF (pro-TNF) was found to be biologically active and can transduce signals to adjacent cells by cell–cell contact [6]. In fact, pro-TNF is superior to mature TNF in activating the human p80 TNF receptor for a number of cellular functions [7]. In addition, recent reports revealed that the pro-TNF functions not only as an activating ligand but also as a receptor that transmits reverse signals from TNF receptor [8]. In human monocytes/macrophages, reverse signaling through pro-TNF confers resistance to lipopolysaccharide [9]. It was also demonstrated that reverse signaling through pro-TNF induced E-selectin expression on activated human CD4$^+$ T-cells [10]. Thus, biological activities of 26 kDa transmembrane TNF are distinct from that of the 17 kDa secretory form of TNF and this characteristic activity of pro-TNF is mediated by the 76 residue leader sequence of this molecule. However, the precise role of this leader sequence on the function of pro-TNF has not been well characterized.

With regard to the mechanism of reverse signaling through pro-TNF, Watts et al. provided evidence that casein kinase I phosphorylates the cytoplasmic domain of pro-TNF at a consensus sequence that is conserved among members of the TNF family [8]. In this study, to reveal the possible involvement of other co- or post-translational protein modifications in the expression and cellular processing of pro-TNF, acylation of pro-TNF was examined by metabolic labeling of the COS-1 cells or Sf9 cells transfected with cDNA encoding pro-TNF. As a result, pro-TNF was found to be palmitoylated at a specific cysteine residue located at the boundary between the transmembrane and cytoplasmic domains.

2. Materials and methods

2.1. Materials

Restriction endonucleases, DNA modifying enzymes, RNase inhibitor, and *Taq* DNA polymerase were purchased from Takara Shuzo, Kyoto Japan. RNase was purchased from Boehringer-Mannheim, Germany. [^{3}H]leucine, [^{3}H]myristic acid, [^{3}H]palmitic acid and Amplify were from Amersham, UK. The Dye Terminator Cycle Sequencing kit was from Applied Biosystems, USA. Anti-human TNF polyclonal antibody was purchased from R&D systems, USA. Protein G Sepharose was from Pharmacia Biotech, Sweden. Other reagents purchased from Wako Pure Chemical, Daiichi Pure Chemicals, and Seikagaku Kogyo (Japan) were of analytical or DNA grade.

2.2. Plasmid construction

Plasmid pBluescript II SK(+) lacking *Apa*I and *Hin*dIII sites was constructed as previously described [11], and designated pB. Plasmid

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Abbreviations: TNF, tumor necrosis factor; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence; DPBS, Dulbecco's phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LAT, linker for activation of T-cells

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pBpro-TNF, which contains the full-length human pro-TNF cDNA, and plasmid pBV2G,R3A-TNF, containing a cDNA encoding the *N*-myristoylated mutant of the mature domain of TNF, were constructed as described [11,12]. Plasmids pBC-47A-pro-TNF and pBC-28A-pro-TNF were constructed by utilizing polymerase chain reaction (PCR). For this procedure, pBpro-TNF served as a template and two mutagenic primers (C-47A, 5′-ATATGGGCCCCAGGGCTCCAG-GCGGGGCCTTGTTCC-TCAGCCTC-3′; C-28A, 5′-ATATGGCGC-CACCACGCTCTTCGCCCTGCTGCACTTT-GGA-3′) and B1(5′-GCCGGGATCCTAGGGCGAATTGGGTACC-3′) as primers. For plasmid pBC-47A-pro-TNF, after digestion with *Apa*I and *Eco*RI, the amplified product was subcloned into pBpro-TNF at *Apa*I and *Eco*RI sites. For plasmid pBC-28A-pro-TNF, after digestion with *Nar*I and *Eco*RI, the amplified product was subcloned into pBpro-TNF at *Nar*I and *Eco*RI sites. Plasmids pcDNA3 containing mutant pro-TNF cDNAs used for transfection of COS-1 cells were constructed as follows; The *Bam*HI/*Eco*RI fragments containing each of the mutant pro-TNF cDNA were excised from each of the pB vectors containing mutant pro-TNF cDNAs and then ligated to the *Bam*HI/*Eco*RI sites of pcDNA3. Construction of recombinant transfer vector (pAcYM1) containing pro-TNF cDNA used for baculovirus expression system was performed as previously described [13]. The DNA sequences of these recombinant cDNAs were confirmed by the dideoxy-nucleotide chain termination method [14].

2.3. Transfection of COS-1 cells and determination of acylated proteins

The simian virus 40-transformed African Green monkey kidney cell line, COS-1, was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (FCS, Gibco BRL). Cells (2×10^5) were plated onto 35 mm diameter dishes 1 day before transfection. pcDNA3 construct (2 μg; Invitrogen, San Diego, CA, USA) containing mutant TNF cDNA was used to transfect each plate of COS-1 cells along with 4 μl of Lipofectamine (2 mg/ml; Gibco BRL) in 1 ml of serum-free medium. After incubation for 5 h at 37°C, the cells were refed with serum containing medium and incubated again at 37°C for 48 h. The cells were then washed twice with 1 ml of serum-free DMEM and incubated for 5 h in 1 ml of DMEM with 2% FCS containing [^{3}H]leucine (30 μCi/ml), [^{3}H]myristic or palmitic acid (100 μCi/ml). In this case, leucine-free DMEM was used for the labeling with [^{3}H]leucine. Subsequently, the cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and collected with cell scrapers, followed by lysis with 200 μl of RIPA buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), proteinase inhibitors) on ice for 20 min. The cell lysates were centrifuged at 15 000 rpm at 4°C for 15 min in a microcentrifuge (Hitachi-CF15D2) and supernatants were collected. The samples were immunoprecipitated with a specific goat anti-hTNF polyclonal antibody (R&D Systems) as described [11] and then analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography.

2.4. Virus and Sf9 cells

AcVAPK6, used as an expression vector in this study, contains *Escherichia coli* β-galactosidase (Lac Z) gene in place of the polyhedrin gene of Autographa californica nuclear polyhedrosis virus (AcNPV). AcVAPK6 and recombinant baculovirus were propagated in *Spodoptera frugiperda* (Sf) cells at 28°C in TC100 medium supplemented with 10% FCS. Sf9 cells were subcultured when the cell density reached $2–2.5\times10^6$ cells/ml, two or three times a week.

2.5. Transfection and selection of recombinant baculovirus

AcVAPK6 baculovirus DNA was linearized by digestion with *Eco*81I to increase the level of recombination. The AcVAPK6 and pAcYM1 with pro-TNF cDNA were cotransfected into Sf9 cells with lipofectin (Gibco BRL). The lipofectin mixture (8 μl lipofectin and 4 μl water) was added dropwise to 12 μl of the cotransfection mixture containing 1 μg of pAcYM1 with pro-TNF cDNA and 50 ng of linearized AcVAPK6 baculovirus DNA, and incubated for 15 min at room temperature. The mixture was inoculated into a 35 mm tissue culture dish containing 1.5×10^6 Sf9 cells in 1 ml of serum-free TC100 and incubated for 4 h at 28°C. 1 ml of TC100 with 10% FCS was then added to the cells, and the supernatant was harvested after a 96 h incubation. Recombinant virus was purified by plaque forming assay. Plaques with a white phenotype were picked up and purified repeatedly until no blue plaques could be detected.

2.6. Determination of acylated proteins expressed in Sf9 cells

Sf9 cells (1.5×10^6) in 35 mm tissue culture dishes were infected with virus at a multiplicity of infection of one plaque forming unit per cell at 28°C. After incubation for 48 h, cells were washed twice with 1 ml of FCS-free TC100 and cultured for 3 h in 1 ml of TC100 with 2% FCS containing [^{3}H]myristic or palmitic acid (150 μCi/ml). The cells were washed three times with NPV-PBS (140 mM NaCl, 26 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4) and then lysed with RIPA buffer on ice for 20 min. The cell lysates were centrifuged at 15 000 rpm at 4°C for 15 min in a microcentrifuge (Hitachi-CF15D2) and supernatants were collected.

2.7. Western blotting

The total cell lysates of each group of transfected cells were resolved by 12.5% SDS–PAGE and then transferred to an Immobilon-P transfer membrane (Millipore). After blocking with non-fat milk, the membrane was probed with a specific goat anti-hTNF antibody as described previously [15]. Immunoreactive proteins were specifically detected by incubation with horseradish peroxidase-conjugated anti-goat IgG antibody (Santa Cruz). The membrane was developed with enhanced chemiluminescence (ECL) Western blotting reagent (Amersham Corp.) and exposed to an X-ray film (Kodak).

2.8. SDS–PAGE and fluorography

Samples were denatured by boiling for 3 min in SDS-sample buffer followed by analysis by SDS–PAGE on a 12.5% gel. Thereafter, the gel was fixed and soaked in Amplify™ (Amersham) for 30 min. The gel was dried under vacuum and exposed to an X-ray film (Kodak) for an appropriate period.

3. Results

3.1. Transmembrane TNF is palmityolated

To determine possible protein acylation of the transmembrane form of TNF (pro-TNF), COS-1 cells transfected with human pro-TNF cDNA were metabolically labeled with [^{3}H]leucine, [^{3}H]myristic acid and [^{3}H]palmitic acid. The labeled pro-TNFs were immunoprecipitated from total cell lysates and subjected to SDS–PAGE and fluorography. V2G,R3A-TNF, an *N*-myristoylated mutant of mature TNF, which is known to be efficiently *N*-myristoylated [12] was subjected to the same procedure. Western blotting of the total cell lysates revealed that TNF was not expressed in control COS-1 cells as shown in Fig. 1B, lanes 2–4. When pro-TNF cDNA was transfected into COS-1 cells, a remarkable amount of 26 kDa pro-TNF and a trace amount of a processed form of TNF (17 kDa) were detected (Fig. 1B, lanes 5–7). The expression of these two TNF species was confirmed by the metabolic labeling with [^{3}H]leucine as shown in Fig. 1A, lane 4. Metabolic labeling with [^{3}H]myristic acid and [^{3}H]palmitic acid revealed that 26 kDa pro-TNF was specifically labeled with [^{3}H]palmitic acid. In the case of V2G,R3A-TNF, efficient expression of 17 kDa TNF was detected by Western blotting (Fig. 1B, lanes 8–10) and by metabolic labeling with [^{3}H]leucine (Fig. 1A, lane 7). Metabolic labeling with [^{3}H]myristic acid and [^{3}H]palmitic acid revealed that V2G,R3A-TNF is specifically labeled with [^{3}H]myristic acid, but no incorporation of [^{3}H]palmitic acid was observed as shown in Fig. 1A, lanes 8 and 9. These results strongly indicated that 26 kDa pro-TNF is not *N*-myristoylated, but palmitoylated.

To confirm palmitoylation of pro-TNF in a different expression system, pro-TNF expressed in Sf9 cells by the baculovirus expression system was examined for protein acylation. As shown in Fig. 2A, 26 kDa pro-TNF was strongly expressed and a trace amount of a processed form of TNF (17 kDa) were detected, as was the case with COS-1 cells

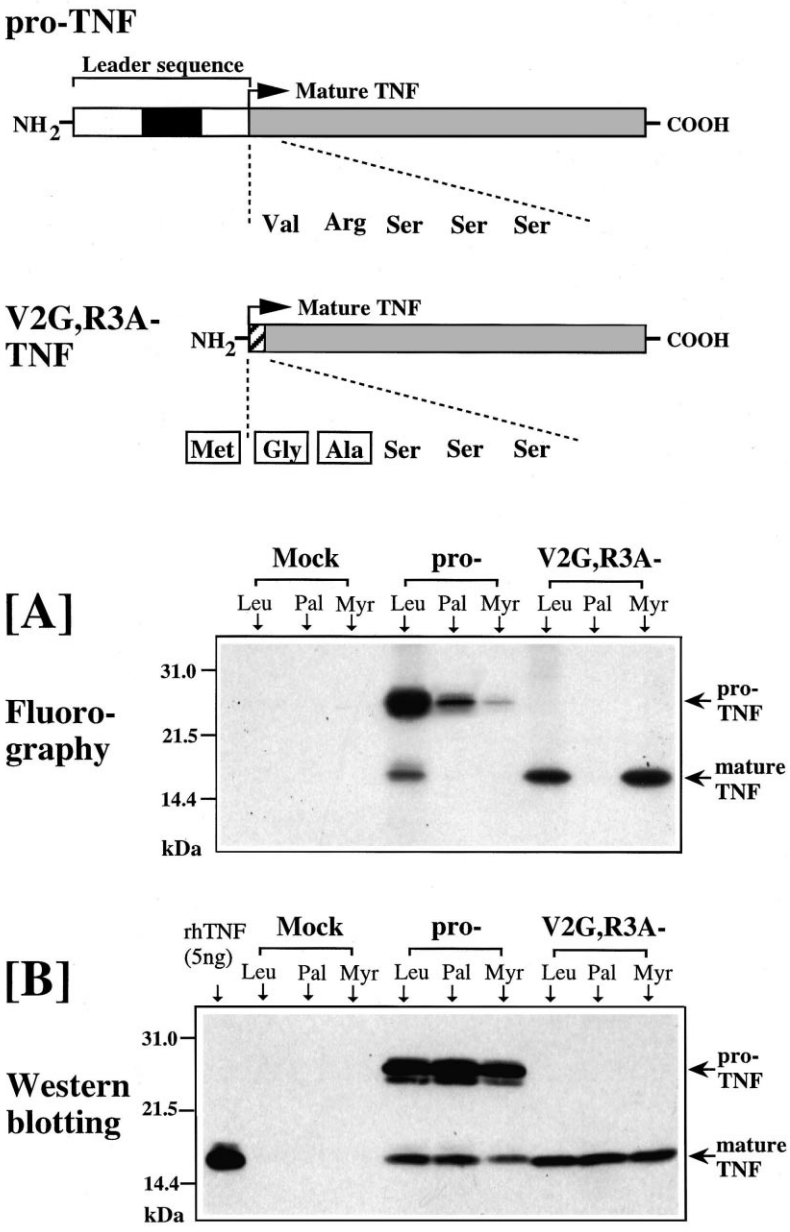

Fig. 1. Transmembrane TNF is palmitoylated. Pro-TNF and V2G,R3A-TNF were expressed in COS-1 cells and the cells were labeled with [^{3}H]leucine (Leu), [^{3}H]palmitic acid (Pal) or [^{3}H]myristic acid (Myr). A: The labeled proteins in total cell lysates of each cells were immunoprecipitated with anti-TNF antibody. Samples were then subjected to SDS–PAGE and fluorography. B: Total cell lysates of each cells were directly analyzed by Western blotting using anti-TNF antibody.

when pro-TNF cDNA was transfected into Sf9 cells. Metabolic labeling with [^{3}H]myristic acid and [^{3}H]palmitic acid revealed that 26 kDa pro-TNF is specifically labeled with [^{3}H]palmitic acid as shown in Fig. 2B. These results clearly indicated that transmembrane TNF expressed in Sf9 cells was also palmityolated.

Next we determined the chemical nature of the linkage between the fatty acid and pro-TNF. To distinguish between a labile thioester linkage and a stable amide bond, we examined whether the [^{3}H]palmitic acid labeling of pro-TNF expressed in COS-1 cells was sensitive to hydroxylamine. As shown in Fig. 3, treatment of the SDS–PAGE gel with neutral 1 M hydroxylamine completely removed both [^{3}H]palmitic acid and [^{3}H]myristic acid labeling of pro-TNF. The fact that the [^{3}H]myristic acid labeling of pro-TNF was sensitive to hydroxylamine suggested that the low level of [^{3}H]myristic acid incorporation could be explained by chain elongation of myristic acid to palmitic acid. In contrast, [^{3}H]leucine labeling of pro-TNF and V2G,R3A-TNF and [^{3}H]myristic acid labeling of V2G,R3A-TNF were not affected by the hydroxylamine treatment. These results indicated that the palmitic acid was esterified to a cysteine residue of pro-TNF.

3.2. *Palmitoylation of pro-TNF occurs specifically at Cys-47, located at the boundary between the transmembrane and cytoplasmic domains of pro-TNF*

Next we established the specific cysteine palmitoylation site of pro-TNF. To detemine whether the palmitoylation site is located at the leader sequence of pro-TNF or in the mature domain of TNF, [^{3}H]palmitic acid labeling of secreted mature

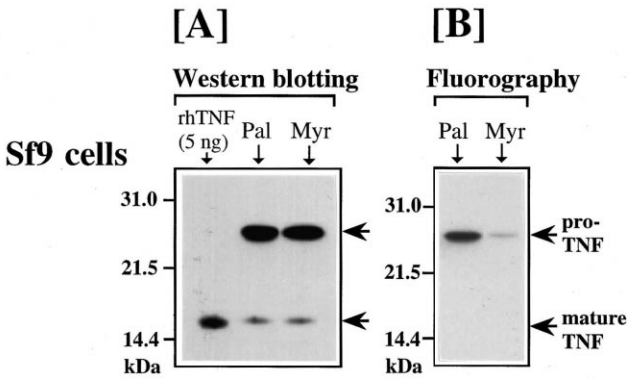

Fig. 2. Transmembrane TNF expressed in Sf9 cells by vaculovirus expression system is also palmitoylated. Pro-TNF was expressed in Sf9 cells by vaculovirus expression system and the cells were labeled with [^{3}H]palmitic acid (Pal) or [^{3}H]myristic acid (Myr). A: Total cell lysates of each cells were analyzed by Western blotting using anti-TNF antibody. B: The labeled proteins in total cell lysates of each cells were immunoprecipitated with anti-TNF antibody. Samples were then subjected to SDS–PAGE and fluorography.

TNF was examined. COS-1 cells transfected with human pro-TNF cDNA were labeled with [^{3}H]leucine and [^{3}H]palmitic acid. The labeled TNF was immunoprecipitated from total cell lysates and cell culture supernatants and then subjected to SDS–PAGE and fluorography. As shown in Fig. 4, lanes 1 and 2, a large amount of 17 kDa mature TNF was found to be secreted in the culture supernatants. However, no [^{3}H]palmitic acid incorporation was observed on this secreted

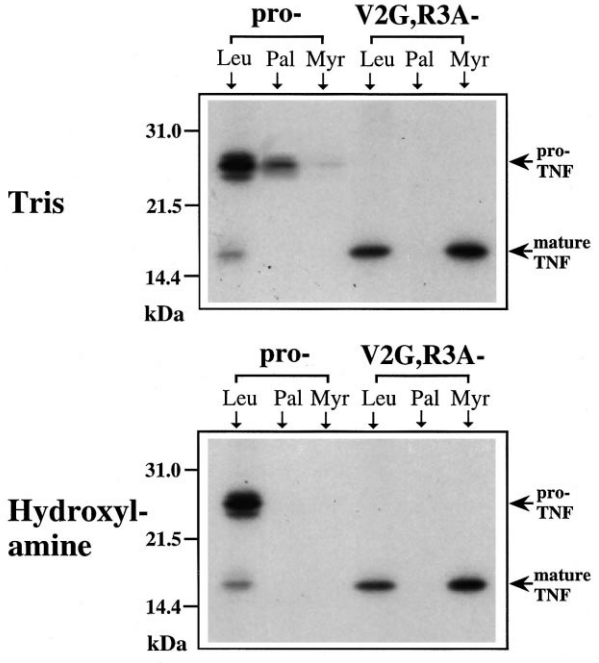

Fig. 3. Palmitic acid is bound to transmembrane TNF through thioester linkage. Pro-TNF and V2G,R3A-TNF were expressed in COS-1 cells and the cells were labeled with [^{3}H]leucine (Leu), [^{3}H]palmitic acid (Pal) or [^{3}H]myristic acid (Myr). The labeled proteins in total cell lysates of each cells were immunoprecipitated with anti-TNF antibody. Samples were then subjected to SDS–PAGE. After fixation with 30% methanol/7% acetic acid for 1 h, the gel was washed three times with distilled water, soaked for 8 h in either 1 M hydroxylamine pH 7.5 or 1 M Tris pH 7.5, then prepared for fluorography.

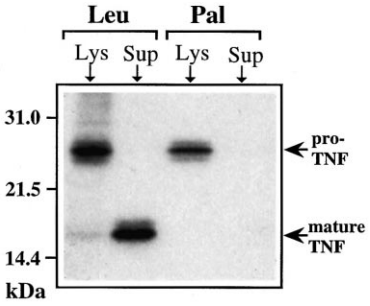

Fig. 4. Palmitoylation site is located in the leader sequence of transmembrane TNF. Pro-TNF was expressed in COS-1 cells and the cells were labeled with [^{3}H]leucine (Leu) or [^{3}H]palmitic acid (Pal). The labeled proteins in total cell lysates or cell culture supernatants of each cells were immunoprecipitated with anti-TNF antibody. Samples were then subjected to SDS–PAGE and fluorography.

TNF molecule (lane 4), indicating that the palmitoylation site is located in the leader sequence of pro-TNF.

Since two cysteine residues (Cys-47, Cys-28) are located in the leader sequence of pro-TNF, we next determined which cysteine residue was palmitoylated. For this purpose, two mutant pro-TNFs, C-47A-pro-TNF and C-28A-pro-TNF, in which Cys-47 or Cys-28 in pro-TNF was replaced with Ala, were generated and their susceptibility to protein palmitoylation was evaluated in the COS cell expression system. Western blotting analysis of total cell lysates and cell culture supernatants of each of the transfected cells revealed that efficient expression of pro-TNF and efficient secretion of mature TNF was equally observed with C-47A-pro-TNF and C-28A-pro-TNF as compared with wild type pro-TNF as shown in Fig. 5A,C. [^{3}H]palmitic acid labeling of these transfected cells revealed that replacement of Cys-47 with Ala completely inhibited the labeling, whereas that of Cys-28 did not affect the labeling (Fig. 5B). These results indicated that Cys-47 of pro-TNF was essential for the attachment of palmitic acid, most likely because the Cys residue was the specific site of covalent attachment of palmitic acid. Thus, site-directed mutagenesis of the two cysteine residues residing in the leader sequence of pro-TNF demonstrated that palmitoylation of pro-TNF occurs solely at Cys-47, located at the boundary between the transmembrane and cytoplasmic domains of pro-TNF.

4. Discussion

A number of eukaryotic cellular proteins are covalently modified with long-chain fatty acids such as myristic and palmitic acids [16–20]. Many of these acylated proteins play key roles in regulating cellular structure and function. *N*-Myristoylation is the result of cotranslational addition of the saturated 14-carbon fatty acid myristate to a glycine residue at the extreme N-terminus after removal of the initiating methionine. A stable amide bond links myristate irreversibly to proteins. Unlike *N*-myristoylation, palmitoylation is a reversible post-translational modification of proteins with the saturated 16-carbon fatty acid palmitate. Palmitate is usually linked to cysteine residues through a thioester bond. The identity of the enzyme responsible for protein palmitoylation has not been firmly established yet. Therefore, neither precise substrate specificity nor the consensus amino acid sequence recognized by

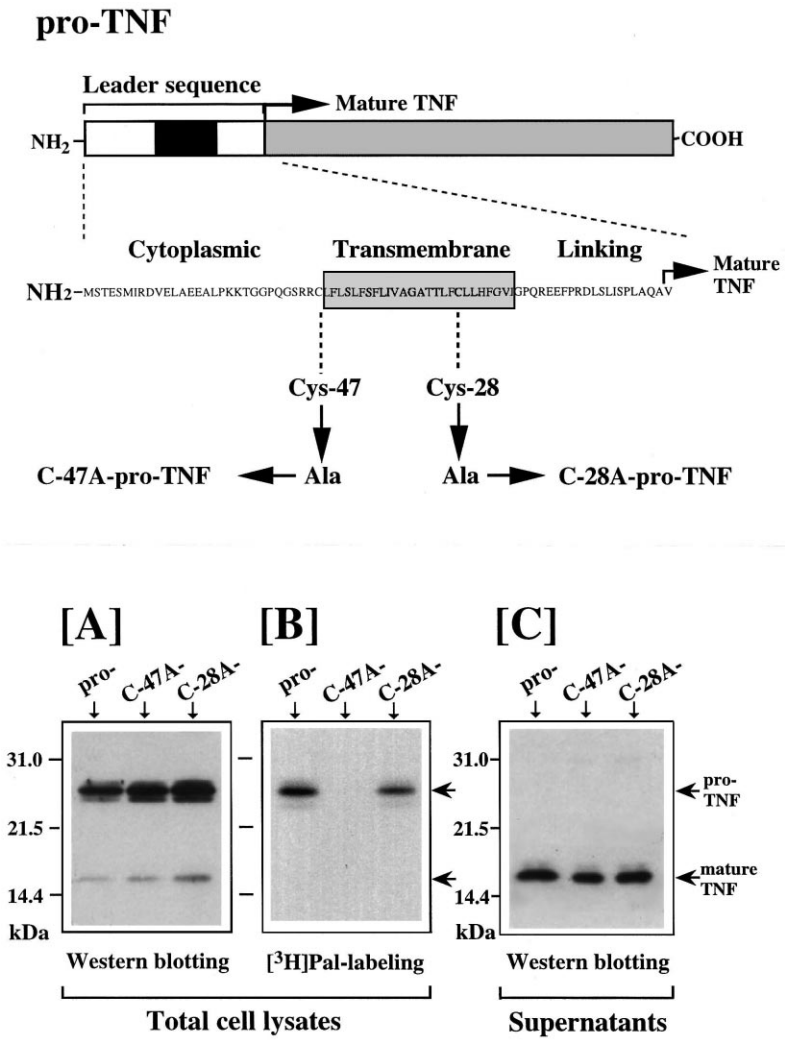

Fig. 5. Palmitoylation of transmembrane TNF occurs specifically at Cys-47. Pro-TNF, C-47A-pro-TNF and C-28A-pro-TNF were expressed in COS-1 cells and the cells were labeled with [^{3}H]palmitic acid. Total cell lysates (A) or cell culture supernatants (C) of each cells were analyzed by Western blotting using anti-TNF antibody. The labeled proteins in total cell lysates of each cells were immunoprecipitated with anti-TNF antibody. Samples were then subjected to SDS–PAGE and fluorography (B).

this enzyme has been elucidated to date. To complicate matters, there are numerous reports documenting the existence of non-enzymatic palmitoylation of proteins.

In integral membrane proteins the acylated cysteine residues are always located at the boundary between the transmembrane region and the cytoplasmic tail of the respective protein [21]. In the present study, we revealed that the human transmembrane TNF was palmitoylated at a specific cysteine residue, Cys-47, located at the boundary between the transmembrane and cytoplasmic domains of pro-TNF, consistent with the previous observations. When the amino acid sequences of 15 pro-TNF molecules derived from different species are surveyed, Cys-47 is conserved in all pro-TNF species, suggesting the critical role of this modification in the physiology of pro-TNF.

Concerning the acylation of pro-TNF, it was previously reported that the 26 kDa pro-TNF was myristoylated in human monocytes and that the acylation occurred via amide bond formation with two specific ε-amino groups of internal lysine residues(Lys-58, Lys-57) present within the 76 residue leader sequence of pro-TNF [22]. In the present study, however, metabolic labeling of COS-1 cells and Sf9 cells transfected with pro-TNF cDNA clearly demonstrated that the pro-TNF was not myristoylated via an amide bond, but was palmitoylated through the thioester bond at a specific Cys residue Cys-47. One possible explanation for this discrepancy is that the difference in the expression of specific lysyl *N*-ε-NH2-myristoyl transferase that is responsible for the myristoylation of lysine residues of pro-TNF in different cell types. Since TNF is expressed in various cell types such as monocyte/macrophages, T-cells, B-cells, neutrophils, astrocytes, endothelial cells, smooth muscle cells etc. [1], the status of protein acylation of pro-TNF might be different depending on the cell types that express pro-TNF.

Physiological roles of protein palmitoylation of soluble cytoplasmic proteins and of peripheral membrane proteins have been well characterized with the Src family of tyrosine protein kinases and α subunits of heterotrimeric G proteins [23–27]. In these proteins, palmitoylation is involved in the reversible membrane binding, specific membrane targeting, as well as regulated transmembrane signaling of proteins. As compared with these soluble proteins, the role of palmitoylation of in-

tegral membrane proteins remains less well-established. In the case of transferrin receptor and luteinizing hormone receptor, palmitoylation has been reported to regulate the rate of internalization of these receptors [28,29]. Therefore it is possible that palmitoylation of pro-TNF is involved in regulation of internalization or recycling of this molecule. It is also possible that palmitoylation affects shedding of pro-TNF, thereby regulating secretion of the mature secretory form of TNF. However, since efficient expression of pro-TNF and efficient secretion of mature TNF was observed with C-47A-pro-TNF, a palmitoylation-deficient mutant of pro-TNF, as compared with wild type pro-TNF as shown in Fig. 5, palmitoylation of pro-TNF may not strongly affect the ER membrane translocation, sorting to the plasma membrane and maturation of pro-TNF.

Another possibility is the role in the signal transduction through pro-TNF. In this regard, interesting observations were recently reported on the role of palmitoylation on the function of a transmembrane protein linker for activation of T-cells (LAT) [30–32]. Mutation of the palmitoylation sites within LAT prevents LAT partitioning to rafts, and reduces LAT tyrosine phosphorylation as well as recruitment of LAT binding proteins to rafts. Since accumulation and assembly of signaling molecules in rafts is a critical step in T-cell signal transduction, protein palmitoylation seems to play an important role in T-cell signal transduction by specifically recruiting the signaling molecules to rafts. Since reverse signaling through pro-TNF was found to operate in T-cells [10], it is possible to speculate that the palmitoylation of pro-TNF might participate in the signal transduction pathway of reverse signaling through pro-TNF.

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